

## Viable but Nonculturable Stage of *Campylobacter jejuni* and Its Role in Survival in the Natural Aquatic Environment

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Conditions influencing the survival of *Campylobacter jejuni* in the natural aquatic environment have been determined. Release of *Campylobacter* spp. into natural waters by animal hosts is postulated to play a key role in the maintenance of viability and transmission of the organism in the environment. Laboratory flask microcosms containing filter-sterilized stream water were used to test *C. jejuni* for the ability to remain viable in simulated natural systems. The microcosms were compared with the biphasic and shaking broth procedures used routinely for growth of *Campylobacter* spp. in the research laboratory. The stream-water microcosms were analyzed to determine effects of temperature and aeration on the survival of a well-characterized *C. jejuni* strain isolated originally from a human campylobacteriosis patient. Morphological characteristics were evaluated by phase-contrast microscopy and scanning or transmission electron microscopy. Survival curves were quantified on the basis of plate counts, epifluorescent microscopy, optical density measurements, and direct viable counts associated with protein synthesis in the absence of DNA replication. A significant difference was observed between results of direct enumeration, i.e., direct viable counts or acridine orange direct counts, and those from spread plate cultures. In all cases, increasing temperature of cultivation resulted in decreased recoverability on laboratory media, due possibly to an increased metabolic rate, as analyzed by CO<sub>2</sub> evolution in the presence of radiolabeled glutamate. Stream water held at low temperature (4°C) sustained significant numbers of campylobacters for >4 months. Microcosms, aerated with shaking, exhibited logarithmic decline in recoverable *C. jejuni*, while stationary systems underwent a more moderate rate of decrease to the nonculturable state. This nonculturable state of *Campylobacter* spp. holds serious epidemiological implications and requires a re-evaluation of methods used to detect or monitor this organism in environmental samples.

Improved methods for the isolation and cultivation of campylobacters have recently revealed the ubiquitous nature of these potentially pathogenic spirilla (3, 11, 25). Despite advances to date, epidemiological investigations of sporadic campylobacteriosis outbreaks have often failed to isolate the causative organisms from the suspected transmission vehicle (10, 28, 29). Detection in aqueous systems poses special problems in concentration and isolation of the organism (16, 27) that may be linked to specific, measurable environmental parameters such as temperature or oxygenation. Information obtained from human point-source outbreaks (18, 21, 27-29), coupled with successful isolation of *Campylobacter* spp. from environmental waters and water supply systems (12, 21, 27), provides insight into the nature of maintenance and transmission of these bacteria.

The veterinary significance of *Campylobacter* spp. was established in 1913 (17) and has since been confirmed by the avian, ovine, bovine, porcine, canine, and feline sources that have been identified and recognized as important reservoirs (3, 11). Animal hosts of *Campylobacter* spp. have been indicated as a source of contamination and may be associated with survival of the organism in nature, since fecal material is shed directly into aqueous environments.

We have examined the ability of *Campylobacter jejuni* strain HC, originally isolated from a human campylobacteriosis patient, to survive in a sterile stream-water microcosm system, for which a variety of environmental conditions were tested. Plate counts were compared with direct viable count (DVC) and acridine orange direct count (AODC) methods to determine whether nonculturable cells

of *Campylobacter* spp. retain viability. Results from the microcosm systems were compared with those obtained for routine laboratory biphasic and broth growth systems. Effects of temperature and aeration were evaluated in terms of transition of the organism to the viable, but nonculturable state. Morphological transition from predominantly spiral cells in logarithmic phase to predominantly coccoid cells in late stationary phase was monitored by dark-phase and electron microscopy, as well as by density gradient centrifugation.

### MATERIALS AND METHODS

**Test strain.** Strain HC was originally isolated from a male human campylobacteriosis patient and was minimally passaged prior to controlled-rate freezing and storage in liquid nitrogen. HC was identified as *C. jejuni* biotype 2 with the standard battery of biochemical tests (26). Oxidase-positive HC produces catalase, reduces nitrate, evolves H<sub>2</sub>S (detected by lead acetate paper), grows in 1% glycine or 3.5% NaCl, hydrolyzes hippurate, and grows at 42°C but not at 25°C. Carbohydrates are neither fermented nor oxidized, while susceptibility to nalidixic acid and resistance to cephalothin are observed. The growth kinetics of this organism, using several growth systems, have been well characterized in our laboratory, making it ideal for survival studies.

**Preparation of seed inoculum.** Stock seeds were thawed rapidly, plated on 5% sheep blood agar plates (BBL Microbiology Systems, Cockeysville, Md.), and subsequently incubated in 5% O<sub>2</sub>-10% CO<sub>2</sub>-85% N<sub>2</sub> at 37°C for 12 to 16 h. The organisms were passaged twice in succession in a biphasic system, previously described (22), to attain uniform, logarithmic-phase growth. Briefly, cell suspensions

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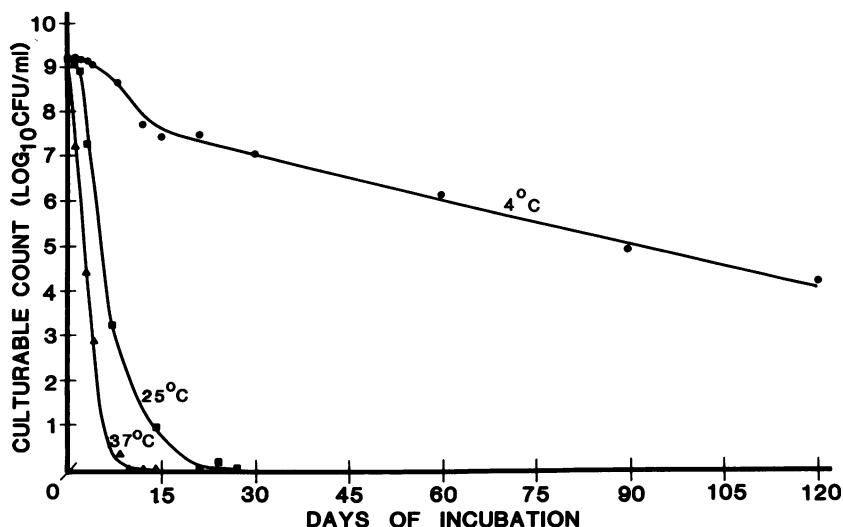


FIG. 1. Survival of *C. jejuni* HC in stationary stream-water microcosms. Data represent mean replicate plate counts (5% sheep blood agar) of replicate samples that had been diluted serially in brucella broth. Microcosms consisted of 50 ml of aged, filter-sterilized stream water in a 125-ml Erlenmeyer flask and were held stationary at 4°C (●), 25°C (■), or 37°C (▲).

were adjusted turbidometrically (optical density at 625 nm = 0.05) in brucella broth (Difco Laboratories, Detroit, Mich.) and incubated over a brucella agar base in a stationary 25-cm<sup>2</sup> tissue culture flask for 12 h at 37°C. Subsequent to the second passage, the organisms were morphologically uniform and were, thereafter, used as inocula in the survival models.

**Survival models.** Three methods were compared to evaluate survival of *C. jejuni* in natural stream water and in artificial growth media. The previously described brucella broth agar biphasic system was compared with a method of cultivation that used a 125-ml Erlenmeyer flask containing 50 ml of brain heart infusion broth (Difco) to which was added yeast extract to a final concentration of 1% (BHI/YE). Both methods have been used successfully in our laboratory to define growth characteristics and were used to evaluate maintenance of culture viability. Replicate flasks that had been previously acid washed and thoroughly rinsed were incubated at 37, 25, or 4°C and were either shaken at 150 rpm or held stationary. Comparable flasks containing filter-sterilized, aged stream water (pH 7.1) constituted our third method, the stream-water microcosm (14, 24). We expected that the stream water utilized in our study would support *C. jejuni* spp. to some extent, as *Campylobacter* spp. have been isolated previously from the aquifer from which the water was obtained. Samples from each system were withdrawn, and the number of cells was quantified at predetermined intervals.

**Enumeration methods.** Turbidometric measurements were made at a wavelength of 625 nm, using a Guilford Stasar III spectrophotometer. Spread plate counts on 5% sheep blood agar were performed in triplicate at appropriate decimal dilutions prepared with brucella broth as diluent. Direct counts were performed (8, 9, 13) with either a Zeiss standard model 18 microscope fitted with an IVFL epifluorescence condenser (DVC) or an American Optical Microstar model 120 fitted with an AO model 2071 vertical fluorescent attachment (AODC). A minimum number of 50 random fields per sample were counted.

Sample volumes of 1 ml for the AODC (8, 9) and 2.5 ml for the DVC (13) were suspended in final volumes of 10 ml of 0.1

M KH<sub>2</sub>PO<sub>4</sub> adjusted to pH 7.2 with NaOH (KNP buffer). These diluted samples were filtered onto 25-mm, 0.2-μm-pore size polycarbonate membrane filters (Nuclepore Corp., Pleasanton, Calif.) that had been previously stained with Irgalan black (9). For the AODC the filters were stained with 0.01% acridine orange and examined by epifluorescence microscopy for cells fluorescing orange. For the DVC, the method of Kogure et al. (13) was used with some modification. The cell suspensions were treated with 32 μg of nalidixic acid per ml to which was added BHI/YE or Casamino Acids broth (Difco) with the same concentration of yeast extract. Following incubation at 42°C for 6 h in the dark, the cells were filtered and the filters were stained and examined as described above.

**Metabolic assay.** L-[U-<sup>14</sup>C]glutamic acid was obtained from New England Nuclear Corp., Boston, Mass., and diluted with its unlabeled counterpart in KNP buffer to provide a final concentration of 20 mM and 0.05 μCi per tube. The evolution of labeled CO<sub>2</sub> from cells incubated at 27 or 37°C was measured as described by Weiss and Westfall (31). A preparation of 100× minimal essential medium vitamins (GIBCO Diagnostics, Madison, Wis.) was added to a final concentration of 1×. Assay inocula were standardized by optical density measurements at 625-nm wavelength and enumerated by spread plate counts on sheep blood agar. Samples of the assay preparation were frozen slowly, and, at a later time, protein determinations were performed by using the Bio-Rad assay (Bio-Rad Laboratories, Richmond, Calif.).

**Sucrose density gradient centrifugation.** Discontinuous sucrose density gradients were prepared with ultrapure sucrose (Bio-Rad Laboratories). The cell preparations were applied in 10% sucrose and the gradients were centrifuged to equilibrium (15,000 rpm, 20 min) in an L2-65B ultracentrifuge (Beckman Instruments, Inc., Palo Alto, Calif.) equipped with an SW-27 swinging-bucket rotor.

**Electron microscopy.** *C. jejuni* samples for scanning electron microscopy were passed through 13-mm, 0.4-μm-pore size Nuclepore filters, washed with KNP buffer, and fixed initially in 6% and subsequently in 3% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2). The filters were washed twice

in additional buffer, postfixed in 1% osmium tetroxide, and dehydrated in graded alcohols for CO<sub>2</sub> critical-point drying. The specimens were mounted on copper tape and sputter coated with gold palladium (60:40) for examination with a JEOL-100CX ASID scanning electron microscope operated at 40 kV.

Specimens for transmission electron microscopy were fixed and dehydrated as above, placed in 1:1 propylene oxide-Luft's epon, and embedded in Luft's epon for thin sectioning. Sections were negative stained with uranyl acetate and lead citrate for examination with a JEOL-100CX electron microscope operated at 80 kV.

## RESULTS AND DISCUSSION

**Effects of temperature on survival and metabolism.** The results obtained in the study reported here indicate that temperature and aeration are two environmental parameters that significantly influence the culturability and, hence, recoverability, of *Campylobacter* spp. (2, 6, 30). Survival in stationary, stream-water microcosms was demonstrated at 37, 25, and 4°C (Fig. 1). *Campylobacters* sampled from microcosms incubated at 4°C and cultured on sheep blood agar plates yielded recoverable populations of  $>10^4$  CFU/ml for up to 4 months. Incubation at 25 and 37°C resulted in decline to the nonculturable state (2, 5, 23, 32) within 28 and 10 days, respectively.

Radiolabeled CO<sub>2</sub> evolution from [<sup>14</sup>C]glutamate substrate was measured (31; H. N. Westfall, D. M. Rollins, and E. Weiss, Appl. Environ. Microbiol., in press) to determine the effect of temperature on the metabolic activity of campylobacters. Spiral forms of *C. jejuni* spp. in logarithmic phase were enumerated, and approximately  $7 \times 10^8$  culturable *C. jejuni* organisms (65 µg of protein) per metabolic assay made up the inoculum. At 37°C, total production and rate of production of CO<sub>2</sub> from radiolabeled glutamate, provided as the sole source of carbon and energy, were high (Fig. 2).

Two separate, distinctly linear rates were generated in the

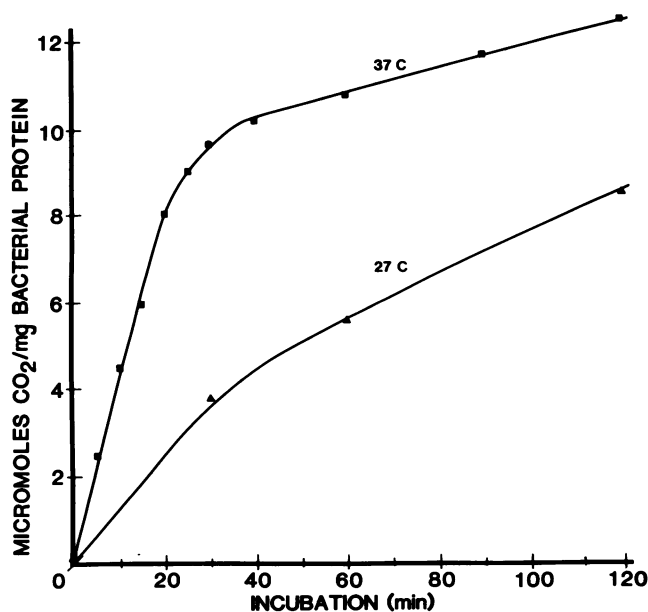


FIG. 2. Effect of temperature on glutamate utilization. *C. jejuni* cells in the logarithmic phase of growth were incubated at 27°C (▲) or 37°C (●) in the presence of [<sup>14</sup>C]glutamic acid. The evolution of <sup>14</sup>CO<sub>2</sub> was measured.

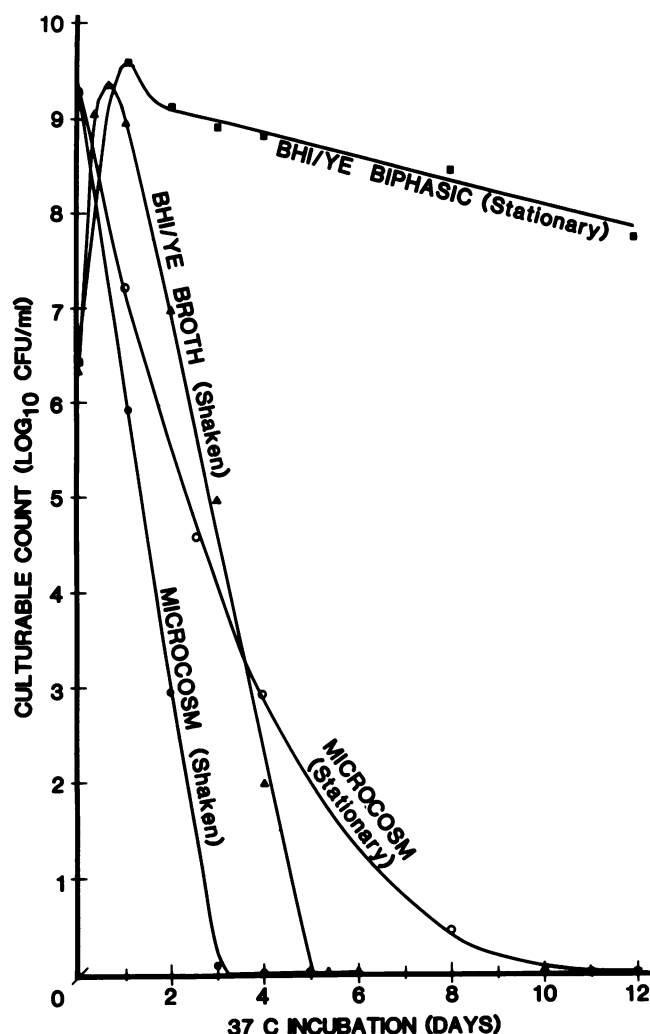


FIG. 3. Comparison of survival in stream water and growth media. Data represent mean replicate plate counts obtained for samples from replicate stream-water microcosms held stationary (○) or rotary shaken at 150 rpm (●); shaken 125-ml Erlenmeyer flasks containing 50 ml of BHI/YE (▲); or biphasic systems with BHI/YE over a BHI/YE agar base (■).

metabolic experiments and were reproducible from experiment to experiment. A rapid linear rate of metabolic activity was followed at 30 to 40 min postinoculation by a moderate, also linear, increase in CO<sub>2</sub> production. The two distinct, linear rates observed for CO<sub>2</sub> evolution were consistently generated and merit further study.

At 27°C, a more moderate rate of metabolic activity was observed (Fig. 2), and the two-phase kinetics of glutamate utilization was less obvious. It can be postulated that, at the higher temperature, glutamate, or any other substrate that might be available in a microcosm, is utilized more rapidly at the expense of other functions, and thus the more rapid decline in culturability.

**Comparative decline to nonculturability in stream water and artificial growth media.** Culturability tested by plate counts of natural stream-water flask microcosms, rotary shaken or held stationary, were compared with those of stationary biphasic or rotary shaken broth cultures of *C. jejuni*. The kinetics of decline in culturability of the shaken

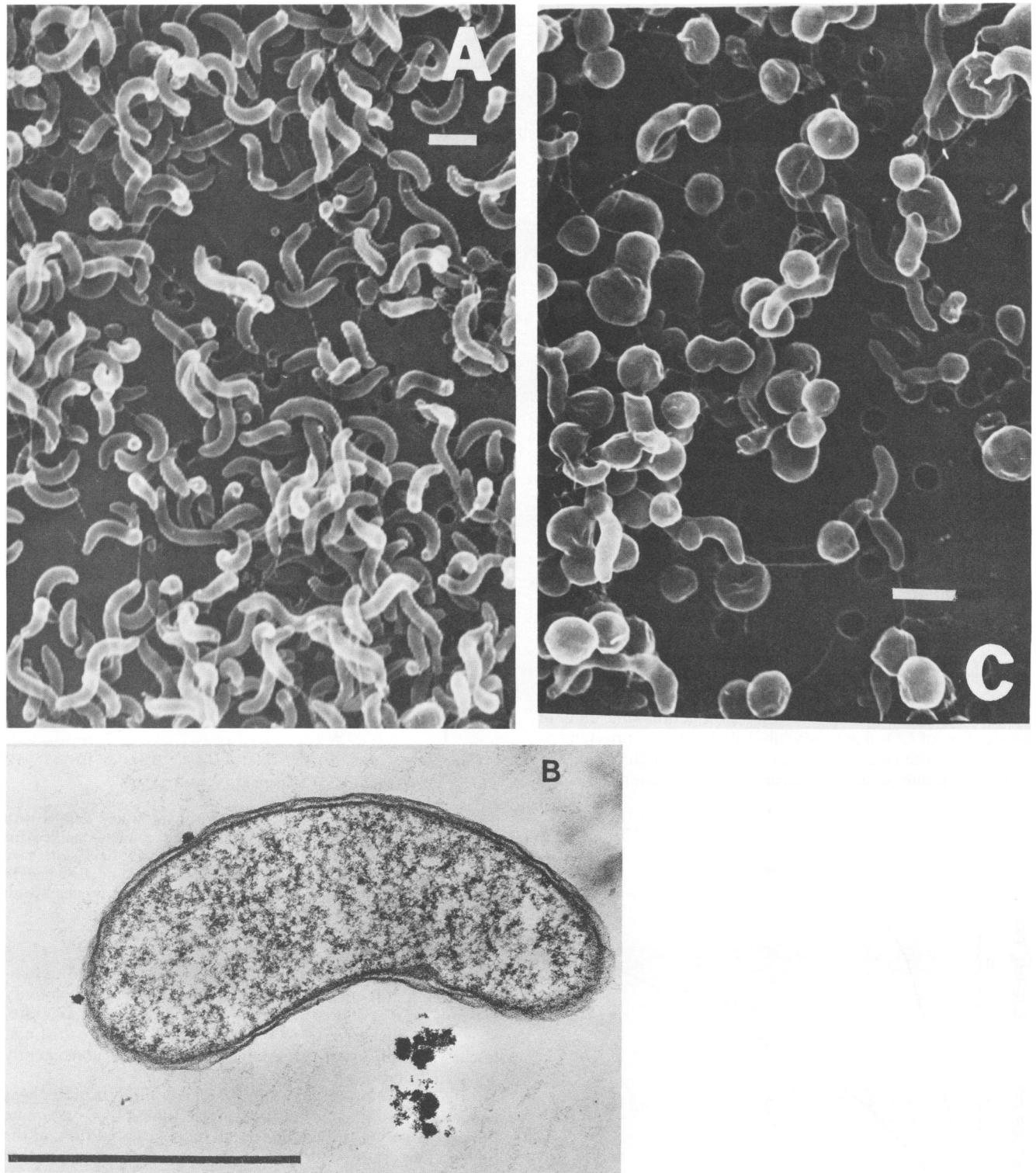
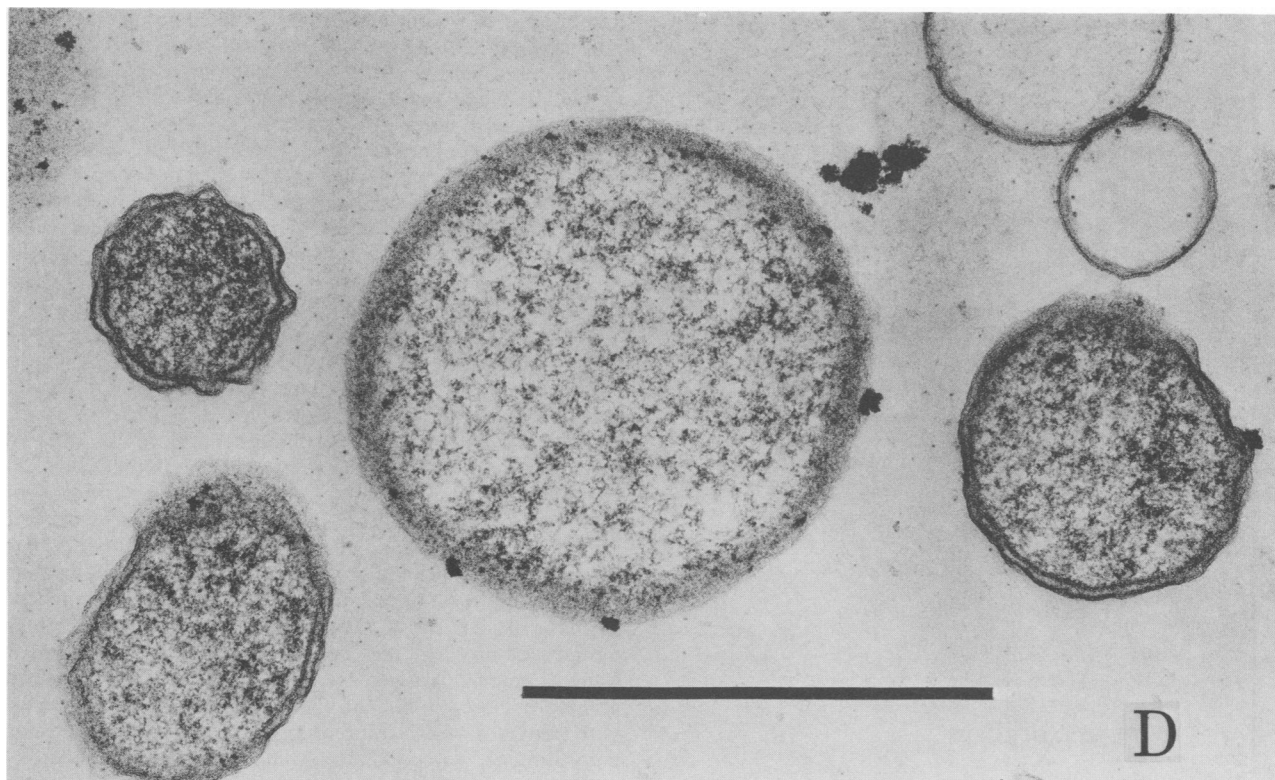


FIG. 4. Electron micrographs of typical campylobacters during exponential growth (A and B) or decline (C and D) phase. (A) Scanning electron micrograph of exponential growth-phase cells filtered through 0.4- $\mu$ m Nuclepore filter; (B) negative-stain transmission electron microscope preparation of single cell during exponential growth; (C) scanning electron micrograph of decline-phase cells revealing spectrum of aberrant forms encountered; (D) transmission electron microscopy of decline-phase *Campylobacter* sp. showing several cells with intact membranes, a spheroplast, and two ghost cells. The latter two forms are presumably nonviable, whereas the more typical coccoid-like cells with intact membranes, though nonculturable, may retain viability. Bar, 1  $\mu$ m.

FIG. 4. *Continued*

cultures, microcosm and broth, were similar (Fig. 3). In the microcosm cultures there was an immediate logarithmic decline to nonculturability in about 3 days. In broth there was a rise in culturable cells for about 1 day followed almost immediately by comparable decline to nonculturability in about 5 days. On the other hand, when the microcosm flasks were held stationary, the rate of decline was much more moderate and nonculturability was attained in about 10 days. In biphasic cultures the cells appeared to be quite stable and only a moderate decline was seen in the 12 days of the experiment shown in Fig. 3. Significant numbers of culturable cells were demonstrated in the biphasic cultures as late as 1 year postinoculation.

**Morphological changes during transition from the culturable to the nonculturable phase.** All of the test systems were inoculated with homomorphous, i.e., morphologically uniform, spiral *C. jejuni* in the logarithmic phase of growth. Repeated examination by dark-phase microscopy revealed the expected gradual transition to a predominance of coccoid forms that is common to both *Spirillum* and *Vibrio* species (5, 7, 15, 19, 20). Transition to the nonculturable form was accelerated at higher temperatures of incubation. Therefore, 37°C was the temperature used for further study of the coccoid forms of *C. jejuni* spp. The majority of these forms maintain an apparently intact, though asymmetric, membrane structure as visualized by scanning and transmission electron microscopy (Fig. 4). Occasional spheroplasts and "ghost" cells can be observed (Fig. 4D) and these forms are believed to be nonviable. Examination of the nonculturable, predominately coccoid forms by electron microscopy showed that cell shape and size varied significantly (Fig. 4B). Transmission electron micrographs often revealed a condensed cytosol in these cells. It was unclear if this condensation represented a survival mechanism or was an artifact

of fixation. Other organisms capable of entering a dormant cycle are known to exhibit similar phenomena (1, 4, 15).

Separation of coccoid from spiral forms was difficult to achieve because of a lack of discrete and complete separation into one or the other morphological type. Rather, there was a continuum of morphological types, with predominance of spiral and coccoid forms, depending on the stage of growth. This phenomenon was documentable by microscopic observation and was corroborated by using discontinuous density gradient centrifugation. Nonculturable, late-stationary-phase *Campylobacter* cells that were centrifuged to equilibrium in sucrose density gradients typically yielded smeared banding in the 30 to 70% sucrose zones, with the heaviest banding noted in 40% sucrose. In addition, a distinct band was observed at approximately 25% sucrose, when the coccoid preparation was centrifuged. This band was subsequently found to contain agar particulates. In contrast, a pellet of motile, spiral, logarithmic-phase cells could be collected at the bottom of the gradient tube and was observed to be evenly dispersed within the 50 to 70% sucrose zone.

Thus, use of density gradient centrifugation was unsuccessful in fractionating the nonculturable forms as a homogeneous coccoid population. In fact, the late decline stage of growth of these organisms is more appropriately described as a nonculturable phase. In view of the results obtained in this study and those reported by Ng et al. (19), the concept of a life cycle of *Campylobacter* spp. that includes a uniform coccoid body stage may be misleading. Sucrose gradient preparations revealed a decrease in density as the campylobacters underwent transition from the spiral to the coccoid form. The late-decline-phase organisms demonstrated a continuous spectrum of cell morphology, evidenced by the 30 to 70% sucrose density preparations.

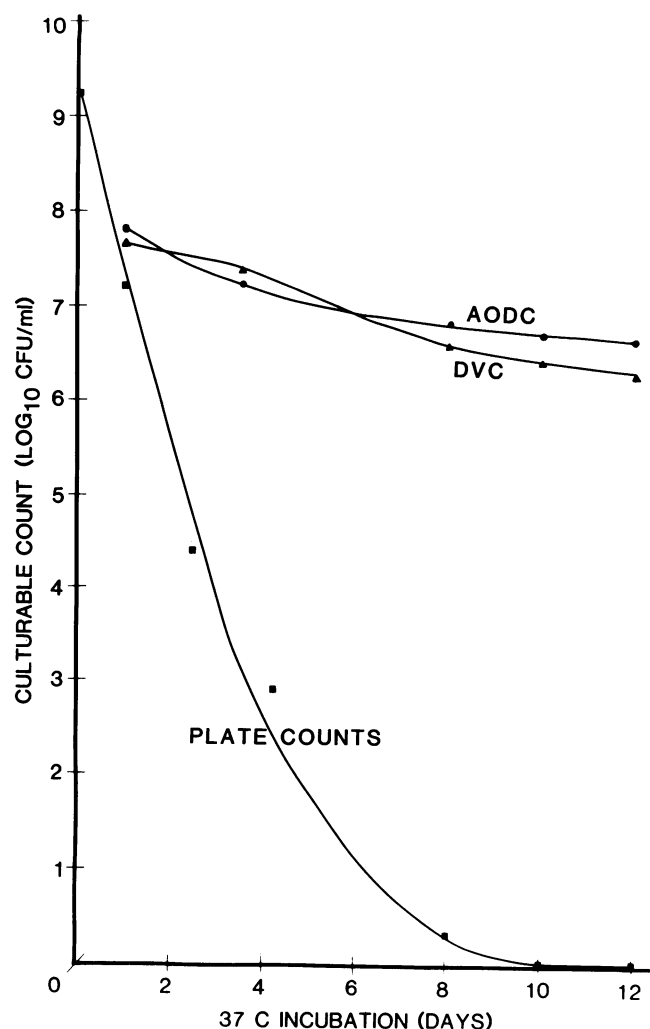


FIG. 5. Quantification of *Campylobacter* viability. Comparison of plate counts (5% sheep blood agar) (■); DVC assaying protein synthesis in the absence of DNA replication (▲); and AODC (●) as indices of viability for stream-water stationary microcosms.

Stationary microcosms typically revealed formation of a viscous layer at the bottom of the flask. The viscous layer appeared to provide a more favorable microenvironment for growth, with respect to oxygen and nutrient concentration. Intact organisms were observed to occur in clumps in this layer, until *Campylobacter* cells in the microcosm were well into the nonculturable phase. The concurrent morphological transitions were associated with the latter phase. A subsequent, relatively abrupt dispersion of the cells with a drop in viscosity and resultant increase in culture homogeneity was consistently observed in all microcosms.

**Survival of *Campylobacter* spp. in the viable, but nonculturable phase.** Our hypothesis that *Campylobacter* spp. survive for extended periods in natural aquifers after deposition by animal hosts led us to explore the viable, but not culturable form in *C. jejuni* since it has been shown to occur in *Vibrio cholerae*, *Salmonella enteritidis*, enteropathogenic *Escherichia coli*, and other waterborne pathogens (5, 23, 32). Three methods currently used to determine viability were compared. The measure of viability generally accepted in microbiology is the spread plate count, a technique that should be defined more accurately as measuring culturability

as opposed to viability. Evidence that nonculturability and nonviability of *Campylobacter* spp. may not be synonymous is presented in Fig. 5. A significant discrepancy was observed between plate and direct counts. The AODC and DVC methods correlated well. As a caution, however, it should be pointed out that direct counting methods are likely to overestimate viability because of occasional background, nonspecific fluorescence, as well as the difficulty in enumerating minute-sized campylobacters in the late stages of growth. The DVC should minimize the latter problem, however, because only elongated cells are counted, i.e., those cells exhibiting continued protein synthesis in the absence of DNA replication and cell division (1, 13). This procedure required the use of a concentration of nalidixic acid sufficient to inhibit DNA replication, which at 32 µg/ml proved adequate, when diluted with BHI/YE or Casamino Acids/YE broth. Surprisingly larger numbers of viable cells were recorded by the DVC than could be detected by using sheep blood agar spread plates. Both direct microscopic methods, i.e., AODC and DVC, yielded results suggesting that  $>10^6$  viable organisms per ml survive after incubation for 10 days, despite the fact that at this time the cells cannot be cultured by spread plating. Preliminary results with animal passage showed that these nonculturable cells retained viability, as has been shown for *V. cholerae* and related, enteric, waterborne pathogens (5). Thus, nonculturability of *Campylobacter* spp. on agar cannot be equated with nonviability. Extrapolating these findings to the natural environment, it is concluded that the present methods used to detect campylobacters do not provide adequate quantification. This hypothesis is corroborated in point-source outbreaks of campylobacteriosis in which no organism can be isolated from the suspected transmission vehicles.

That the *Campylobacter* decline phase of growth is morphologically and culturally consistent in a variety of systems suggests a common transitional pattern associated with suboptimal environmental conditions. The net result is that the campylobacter becomes nonculturable by the routine laboratory culturing methods. The apparent increase in viscosity observed to occur as the organisms "partition out of suspension" and undergo transition from the spiral to the coccoid form is of special interest. The importance of a solid/liquid interface to *Campylobacter* survival merits consideration. Production of an extracellular viscous material may be an adaptation to ensure extended survival in dilute aqueous environments or at such interfaces. The benefits for the organism that can be hypothesized are related to the organism being, thereby, able to control oxygen, nutrient, and metabolite concentrations, as well as the proximity of other organisms. As consistent as the production of this zone was, so also was the dispersion of these clumped bacteria and concurrent decrease in viscosity in the late stages of nonrecoverability. Under proper conditions, the nonculturable forms can be transformed from the viable but nonculturable state to the culturable state. Preliminary results show that animal passage affects this revival.

The evidence obtained to date indicates clearly (Fig. 3) that culturability declines logarithmically in both oligotrophic stream-water microcosms and initially eutrophic shaking broth systems, resulting in the rapid production of nonculturable, predominantly coccoid forms. The biphasic culture system, with a solid/liquid interface, provides an adequate substratum to maintain culturability for extended periods. Interestingly, the viscous mat formed at the agar/broth interface persists until it is mechanically disrupted, thereby providing a microenvironment for prolonged

survival. The importance of the agar/broth interface is attested to by the fact that none of the broth systems maintained survival even after incubation for 2 weeks.

When stream-water microcosms were shaken to induce aeration, culturability declined logarithmically (Fig. 3) to the nonculturable state, i.e., within 3 days. When aeration was confined to passive surface absorption, the decline was significantly less rapid. Since *Campylobacter* spp. are microaerophilic, these results were not unexpected. However, extrapolation to the natural environment suggested that rapidly moving, highly oxygenated water would not support the recoverability of campylobacters as efficiently as quiescent or stagnant, low-oxygen waters.

Results of the temperature studies (Fig. 1) confirm the results reported by Blaser et al. (2) and indicate that campylobacters may maintain culturability in stream water at 4°C for extended periods. This characteristic, perhaps, enables *Campylobacter* spp. to overwinter in cold, slow-moving, low oxygenated aquifers, thus enhancing the opportunity to recycle through animal hosts in the spring months when the temperature rises and the organisms become more metabolically active (Fig. 2). As the temperature increases, viability is best confirmed by DVC and AODC, because culturability significantly declines and, therefore, detection becomes more difficult (Fig. 5). Certainly, growth at 37°C represents standard laboratory conditions, but at this temperature the transition to the nonculturable state increases logarithmically. The brief logarithmic, minimal stationary, and rapid decline phases observed for 37°C broth cultures suggest that 37°C incubation may yield erroneous results if the growth kinetics are not monitored adequately. The "decline" phase begins almost simultaneously with maximum culturability and rapidly moves through the transition phase previously discussed. Cultures utilized at these stages certainly must be considered highly transitional, and these unusual growth kinetics may explain the difficulties observed in repeating results obtained with *Campylobacter* spp., as reported by various investigators.

In conclusion, the viable, but nonculturable stage reported here for *Campylobacter* spp. is significant for understanding the epidemiology of campylobacteriosis. Although the methods described herein offer a means of detection and enumeration of viable but not culturable campylobacter, it is clear that methods presently used for detection and enumeration must be re-evaluated and new techniques must be devised. The strategy of survival demonstrated by *Campylobacter* spp. under adverse environmental conditions has proven to be intriguing and describes a phenomenon perhaps fundamental in microbial ecology.

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